Association of an Unusual Strain of *Xanthomonas campestris* with Apple

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We thank the following individuals for contributions to this work: W. Wergin and R. Lawson, USDA, Beltsville, MD, for SEM and TEM micrographs, respectively; and O. Broome, USDA, Beltsville, MD for Fig. 1A.

Accepted for publication 11 October 1984.

ABSTRACT

Maas, J. L., Finney, M. M., Civerolo, E. L., and Sasser, M. 1985. Association of an unusual strain of *Xanthomonas campestris* with apple. Phytopathology 75:438-445.

A yellow, Gram-negative bacterium was isolated from damaged apple explants in tissue culture (TC). Explants in TC frequently became necrotic, the necrosis involving leaf tips or whole aerial portions of the plants. The bacterium was consistently isolated from affected explants and from greenhouse-grown and nursery plants. The strains were recovered from inoculated apple explants in TC. Symptoms and bacterial strain characteristics agreed with those originally observed. Based on growth on diagnostic media, conventional biochemical and physiological tests, and fatty acid analyses the strains represent an unusual form of *Xanthomonas campestris*. Greenhouse inoculation tests were negative for pathogenicity to tomato, tobacco, cassava, peach, and grape. Apple explants grown in the greenhouse were leaf-inoculated and root-inoculated. Symptoms did not develop on apple; however, the xanthomonad was recovered from leaves 21 and 30 days after leaf inoculation and from stems 60 days following root inoculation. Similar strains were also isolated from apparently healthy bud material from other uninoculated greenhouse-grown apple plants and bud material sent from a commercial nursery. The strains of *Xanthomonas* from apple are apparently pathogenic only in TC; however, they are capable of limited colonization of apple tissue under greenhouse conditions without causing apparent symptoms. We conclude that these strains represent an unusual form of *X. campestris*. These strains differ from other pathovars of *X. campestris* in their either lack of virulence or low virulence and ability to colonize apple tissue without causing apparent symptoms.

Additonal key words: *Malus sylvestris*, *Xanthomonas campestris* pv. *pruni*.

Bacteria, identified as strains of *Xanthomonas campestris*, were isolated from apple explants in tissue culture (TC) and subsequently from apparently healthy buds of apple (*Malus sylvestris* Mill. var. *domestica* (Borkh.)). Based on several investigations, xanthomonads can exist on and in asymptomatic (10,16) and presymptomatic host tissue (8) and on leaves of nonhost plants (9,10,11). Apple leaves and buds may support a diversified microflora (1,2,12). This is apparently the first report of naturally occurring xanthomonad strains from apple. Preliminary characterization, identification, and epidemiology of these strains are presented.

MATERIALS AND METHODS

Tissue culture propagation of apple. Apple buds selected for TC were excised from stems, and the outer bud scales were removed. Prepared buds were washed in 0.01% Tween-20, agitated 5 min, rinsed in sterile distilled water (SDW) three successive times, immersed in 50% calcium hypochlorite plus 0.01% Tween-20 and agitated 20 min, and rinsed three successive times in SDW for 1 min (each rinse was followed by a 5-min wash in SDW with agitation). After disinfestation, emerging leaves were aseptically removed sequentially to expose the apical dome plus two or more leaf primordia that comprised the meristem-tip explant. Explants were established on a solid medium (4). After explants developed leaves (3–4 wk), they were transferred to a proliferation medium (4) then to an establishment medium. During proliferation, new shoots developed from the original explants. The new shoots were removed and either put into fresh proliferation medium or transferred to a rooting medium (4). After 3–4 wk in this medium, rooted explants were established in potting medium following acclimatization in transition from laboratory to greenhouse.

The original apple (cultivar Redchief) budwood from which TC plants were established was received from a commercial nursery in Michigan in 1981. Part of the bud material was grafted onto rootstocks and grown in the greenhouse for 1 yr, and the remainder was held in cold storage for several months prior to establishing it in TC.

Isolation and preliminary characterization of the pathogen. Buds, water-soaked lesions, and necrotic tissue from diseased explants in TC were macerated in SDW; streaked onto plates containing 0.8% nutrient broth, 0.1% yeast extract, 0.5% glucose, and 1.5% agar (NYGA, Difco); and incubated for 48 hr at 26 C.

The following tests were used to characterize the strains: colony characteristics and rate of growth on NYGA and growth at 4, 25, 30, 37, and 41 C on yeast-salts (YS) medium (6); occurrence of a yellow, water-insoluble pigment on yeast extract-dextrose-$CaCO_3$ medium (YDC [17]); Gram stain; catalase (5) and oxidase (API oxidase test kit, API Analytab Products, Plainview, NY) reactions; proteolytic (6), lipolytic (Tween-20 and Tween-80 hydrolysis [19]) and amylolytic capability (6); aesculin hydrolysis (5); acid production from carbohydrates (arabinose, glucose, and mannose [5]); gelatin liquefaction (5); sodium hippurate hydrolysis (5); nitrate and nitrite reduction (5); and urease production (5).

Bacterial strains identified as *Xanthomonas* were examined for size, shape, and flagellation by light microscopy (silver impregnation stain [3]) and transmission electron microscopy (TEM). For TEM examination, cytochrome C in phosphate buffer was applied in a monolayer on grids prior to smearing them with bacterial cells and staining with phosphotungstinate. Isolates were grown on NYGA for 48 hr prior to treatment. Scanning electron microscopic (SEM) examination of infected tissues was done to determine the location of bacteria in tissues.

Cellular fatty acid analyses. Modified methods (14) of Moss (15) were used for analysis of total cellular fatty acids. Bacteria were streaked on trypticase soy agar and grown at 28 C for 48 hr. One 4-mm-diameter loopful of bacteria was added to a 13 × 100-mm screw-cap test tube containing 1.0 ml of 1.2 N NaOH in 50%

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aqueous methanol, capped with a Teflon-lined cap, and saponified for 30 min at 100 C. Samples were cooled to room temperature and acidified to pH 2 with 0.5 ml of 6 M HCl. Samples were methylated with 1.0 ml of 12% BCl_3 reagent (Supelco, Inc., Bellefonte, PA), placed in a water bath at 85 C for 5 min, then cooled to room temperature. Fatty acid methyl esters were extracted by adding 1 ml of a mixture of hexane and diethyl ether (1:1), gently mixing for 3 min, and removing the lower (aqueous) phase. Samples were washed by adding 3.0 ml of 0.3 M NaOH. Tubes were capped and mixed end-over-end five times. The upper (organic) phase was removed to a gas chromatography vial and either analyzed immediately or capped with a fluoroelastomer cap and stored frozen. Fatty acid methyl esters were analyzed with a Hewlett-Packard (H-P) model 5790 gas chromatograph equipped with a flame ionization detector, an autosampler, an H-P 3390A Integrator, and an H-P 85 Computer. The 25-m-long glass capillary column was coated with crosslinked 5% phenylmethyl silicone.

Propagule decontamination of surface bacteria. Incyte (Alcide Corp., Westport, CT), a chlorine dioxide complex, was used in another sterilizing procedure for surface decontamination of propagation shoots taken from apple explants. Incyte is a broad-spectrum decontaminating material (prepared by mixing part A and part B liquids just before use) that is nontraumatic to plant tissue. Eighty shoots were removed from explants infected by *Xanthomonas* in the proliferation medium, and their leaves were removed prior to treatment. Shoots were washed in SDW followed by immersion for 5 min in a mixture of Incyte and part A, part B, and SDW (1:1:4) adjusted to pH 6.5 as specified by instructions from the supplier. Shoots were then transferred to a liquid proliferation medium containing Incyte (part A, part B, and proliferation medium [1:1:500]) in ten 125-ml flasks which were placed on a rotating wheel for 24 hr at 20 C. Shoots were then placed on solid proliferation medium without decontaminating material.

Pathogenicity tests. *Tissue culture.* Unrooted proliferated explants of Triple Red Delicious and of a McIntosh seedling about 1.5 cm long were inoculated by wounding the distal portion of each stem once with a 0.45-mm (26-gauge) needle dipped into a bacterial cell suspension containing 10^8 colony-forming units (cfu) or by dipping apical portions of unwounded stems into an inoculum suspension containing 10^8 cfu. An apple strain of *Xanthomonas* (XCM-1) that was used in these experiments was originally isolated from naturally infected TC plants. Controls consisted of plants inoculated in the same manner with heat-killed (100 C for 20 min) bacterial cells. Inoculated and control plants were placed into either fresh proliferation or rooting media in glass tissue culture vials (2.5 cm diam × 9 cm long) containing 15 ml of medium. Explants in the proliferation medium were allowed to grow for 3–4 wk, subdivided, and transplanted into fresh rooting medium. All plants in rooting medium were incubated for 7 days at 20 C in darkness before they were examined for symptoms. Water-soaked lesions; necrotic tissues; apparently healthy tissue from stems, leaves, callus, and roots; and culture medium from around roots were plated on NYGA and examined for bacteria and confirmation of the pathogen's identity.

Inoculation of test plants. Leaves of peach (*Prunus persica* (L.) Batsch), cassava (*Manihot esculenta* Crantz), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), and grape (*Vitis vinifera* L.) were inoculated with XCM-1 (10^6 and 10^8 cfu/ml) and *X. pruni* (10^8 cfu/ml) by using a 3-ml sterile, disposable syringe equipped with a 0.46-mm-diameter (26-gauge) needle by injecting the three youngest leaves of each plant with bacterial suspensions until the area appeared to be water-soaked. Heat-killed bacterial suspensions were used as controls. Undersides of the apple (cultivar York) leaves were inoculated at five sites on each side of the midvein with XCM-1 (10^8 cfu/ml) and a heat-killed suspension of XCM-1 by air brush at 138 kPa (20 psi) until the inoculated area appeared to be water-soaked. Plants were kept in a greenhouse under a shade cloth for 7 days, except apple plants which were observed for 14 days (under shade). Inoculated areas were sampled, surface sterilized with 0.5% sodium hypochlorite, and plated on NYGA for recovery of XCM-1.

Inoculation of apple explants in the greenhouse. Ten plants each of apple cultivars Paladino Spur McIntosh, Delicious, and Redspur Delicious and six plants of Redchief were inoculated with an air brush. Plants were approximately 5 cm tall and each had

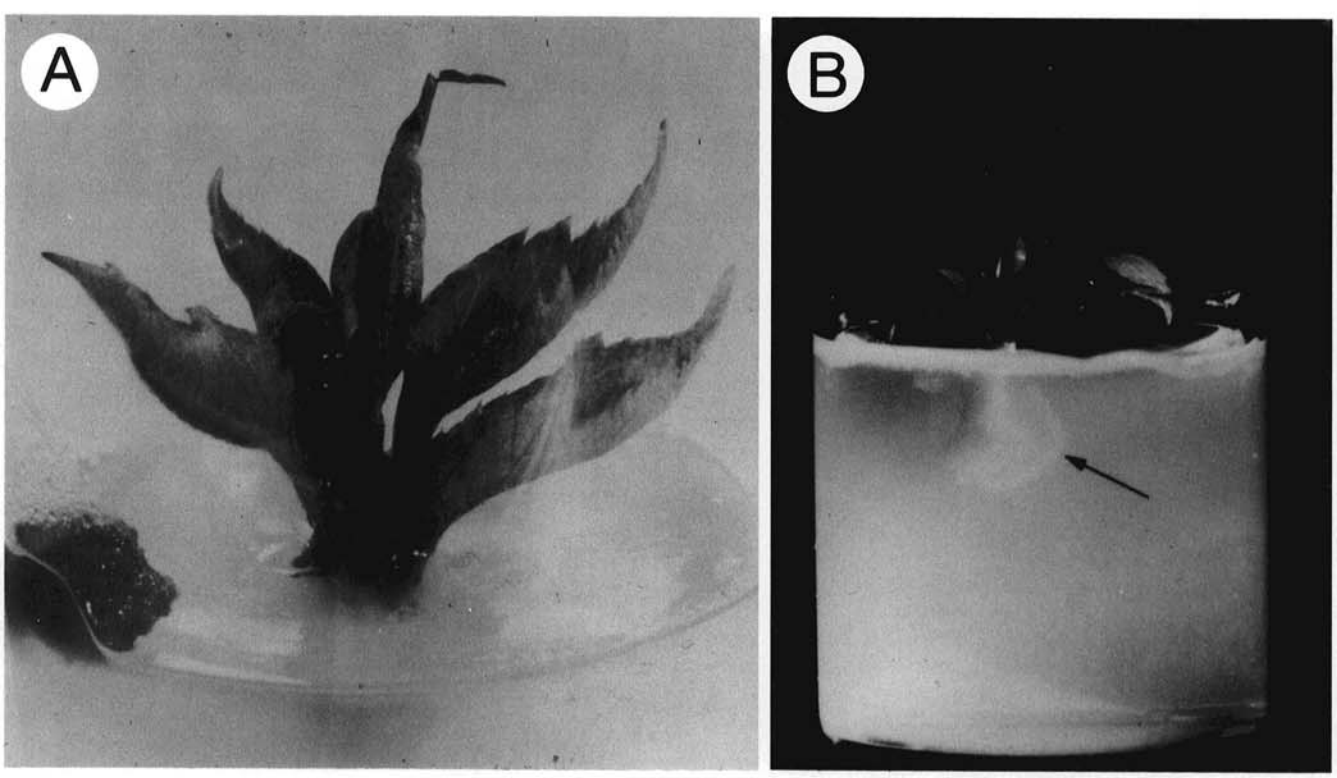

Fig. 1. Apple explants with associated *Xanthomonas campestris* (apple strain) in tissue culture. **A,** Leaf and stem necrosis (courtesy O. C. Broome). **B,** The basal stem of the plant is highlighted to show the characteristic halo of bacterial growth in the medium surrounding the stem (arrow).

three to five leaves. Two to three young leaves were inoculated at two or three sites on the underside of each half of each leaf. The inoculum concentration was 10^6 cfu/ml prepared from cultures of XCM-1 grown on NYGA for 48 hr. Air brush pressure of 138 kPa (20 psi) was used, and the brush was held in contact with the leaf surface until the area showed water-soaking. One-half of each leaf was inoculated, and the other half was treated in the same manner but with SDW as a control. Uninoculated plants served as a second control series. Inoculated plants were covered with plastic bags and shaded for 24 hr then placed on a greenhouse bench with the bags removed. Preliminary samples of leaves were taken 2 wk after inoculation from inoculated and control locations of randomly selected leaves from each cultivar. Individual tissue samples were surface sterilized in 95% ethyl alcohol, macerated in SDW, and spread over three NYGA plates with a flame-sterilized bent glass rod. Colonies were counted after incubation for 3 days. Since XCM-1 was isolated from the inoculated areas and none from the control areas, all leaves were harvested 4 wk following inoculation for recovery of XCM-1. The original inoculation areas were 6 mm in diameter (28.3 mm^2), so an area 6 mm in diameter including the inoculation area was taken randomly from each half of each leaf with a sterile cork borer. Tissue samples were sterilized in 95% ethyl alcohol and ground with 10 ml of SDW in a sterile mortar. Aliquots (0.1 ml) were spread over the surface of each of three petri plates (10-cm diameter) containing NYGA. Plates were incubated at 23 C for 3 days, and colonies determined to be XCM-1 were counted. Randomly selected colonies of XCM-1 were subcultured for fatty acid analysis to verify their identity.

Movement of XCM-1 in apple explants. Explants of apple cultivars Delicious, Redspur Delicious, Paladino Spur McIntosh, and Gala rooted in peat pots were root-inoculated to determine the systemic movement of XCM-1 into stem tissues. Plants were inoculated either by dipping the entire, unwounded root system into a XCM-1 cell suspension (10^8 cfu) or wounded prior to dip inoculation by cutting off a portion of the peat pot and root system. SDW controls were included for each treatment. Numbers of plants varied for each treatment because of availability. Five plants each of Delicious, Redspur, Delicious, and Paladino Spur McIntosh; eight of Spartan; and 10 of Gala were used for the wound inoculation and wound control treatments. Five plants of all cultivars except Gala (10 plants) were dip-inoculated without wounding. Three plants of Delicious, Redspur Delicious, and Paladino Spur McIntosh; five of Spartan; and 10 plants of Gala were included in the dip-only control treatment. Plants were incubated at 20–26 C on a greenhouse bench. Plants were examined after 60 days following inoculation for presence of XCM-1 in roots and stems. Root systems were removed from each plant, washed, weighed, surface sterilized in 0.5% sodium hypochlorite for 2 min, and ground in a sterile mortar with SDW (10 ml SDW per 1.0 g [wet weight] of root tissue). A loopful (4-mm diameter) of macerate was streaked onto each of three petri plates containing NYGA plus cyclohexamide (0.1 mg/ml) (NYGAC). Stems were stripped of leaves and surface sterilized by dipping them in alcohol and flaming followed by immersion in 0.5% sodium hypochlorite for 5 min with constant agitation. The basal 5 cm of each stem was cross-sectioned serially into 2-mm increments, and the sections were plated on NYGAC. All plates were incubated at 24 C for 3 days and examined for the presence of XCM-1.

Colonization of apple leaves. Leaves of young Gala explants grown in the greenhouse were inoculated with an air brush at 138 kPa (20 psi) with XCM-1 suspensions in SDW. Leaves were inoculated at eight sites, four on each side of the midrib. Inoculum consisted of 10^8 cfu/ml (Experiment A) and 10^6 cfu/ml (Experiment B) in the first and second experiment, respectively. Eight leaves were inoculated in Experiment A and 12 in Experiment B. Each leaf was sampled by removing a disk of tissue containing an inoculation site at 0, 1, 2, 3, 4, 7, 10, and 14 (Experiment A) and 0, 1, 3, 7, 10, 14, and 21 (Experiment B) days

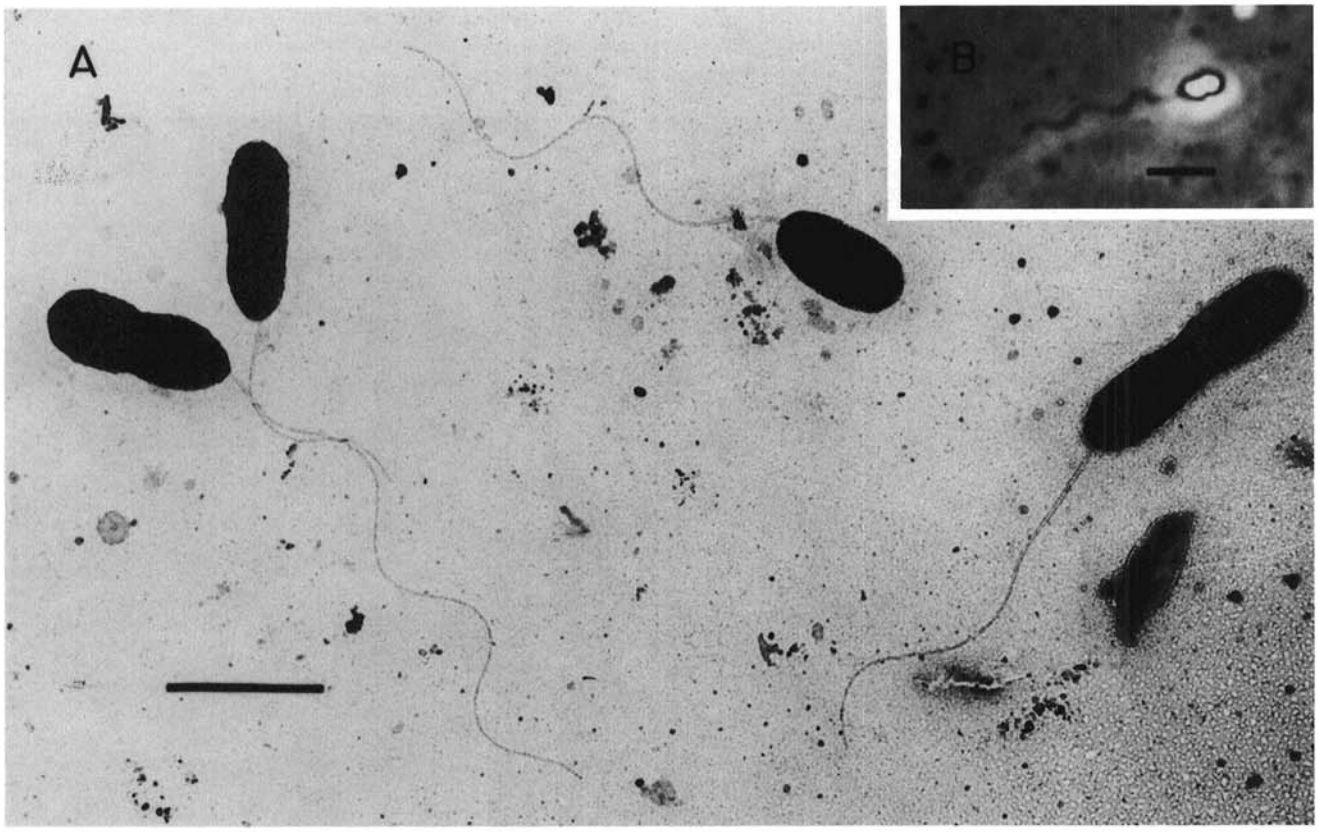

Fig. 2. *Xanthomonas campestris* (apple strain) cells with single, polar flagella. **A,** Transmission electron micrograph (TEM) shows the slightly roughened bacterial cell surface. Scale bar = 1 μm. Courtesy of R. Lawson; **B,** light micrograph (LM) of cell stained with silver nitrate to show the length of the flagellum and to corroborate TEM observations. Comparison of flagella shown in TEM and LM suggests that flagella were either damaged or altered by the TEM process. Scale bar = 2 μm.

following inoculation. Samples were surface sterilized as described above and plated for XCM-1 recovery. Plants were incubated on a greenhouse bench at ambient temperatures (20–28 C) for September-November and given a 16-hr photoperiod with supplemental incandescent light at 100 lux.

RESULTS

Description of disease. Damaged plants in the TC rooting medium were characterized by water-soaked lesions forming on apical portions of stems and leaves of explants. Lesions progressively became brown, necrotic, and desiccated (Fig. 1A). Generally, a halo of bacterial growth developed in the culture medium surrounding the base of the stems (Fig. 1B); however, the young roots apparently were not killed by the bacteria. The disease occurred in 5% or less of the total explant cultures in the rooting medium; however, it often occurred with a high frequency (70–100%) among stems that were taken from the same infected parent explant in the proliferation medium. Stem and leaf symptoms did not occur on plants in the proliferation medium, although the bacterium was isolated from these plants.

TABLE 1. Comparison of growth and biochemical characteristics of pathovars of *Xanthomonas campestris* with those of the apple isolates of *X. campestris*

Characteristic	Reaction	
	X. campestris pathovars[a]	Apple strains
Yellow mucoid colonies on nutrient agar with glucose	+	+
Yellow, water-insoluble pigment	+	+
Colonies 1–3 mm in diameter after 3–5 days	+	+
Growth at 25, 30, 37 C, but not at 4 or 41 C	+	+
Gram-negative rods	+	+
Mottle: single, polar flagellum	+	+
Catalase-positive	+	+
Oxidase-negative or delayed (15–60 sec)	+	+
Protease (casein)	+	+
Amylase	variable	−
Lipase (Tween-20, Tween-80 hydrolysis)	+	+
Aesculin hydrolysis	+	+
Acid produced from carbohydrates (arabinose, glucose, mannose)	+	+
Gelatinase activity	variable	weak +
Sodium hippurate hydrolysis	−	−
Nitrate-to-nitrite reduction	−	−
Nitrite reduction	−	−
Urease production	−	−
Hypersensitivity		
Tomato	+[b]	−
Tobacco	+[b]	−
Pathogenicity to apple	−	+

[a] Data from Dye (5,6) and Dye and Lelliott (7).
[b] For *X. campestris* pv. *pruni*.

Isolation and preliminary characterization of the pathogen. A bacterium that produced small, yellow, mucoid colonies on YDC medium was consistently isolated from diseased apple tissue in TC. The original strain (XCM-1) was isolated from diseased Redchief explants in the TC rooting medium on 18 August 1982. Other strains obtained were: XCM-2, from a McIntosh seedling explant in TC that was inoculated with isolate XCM-1; XCM-3, isolated in 1983 from a bud of an apparently healthy Redchief plant grown in the greenhouse for 1 yr, that had been established by bud graft with the original bud material that yielded XCM-1; and XCM-4, from the same source as XCM-3. A fifth isolate was obtained in 1983 from Redchief budwood sent from the same commercial nursery that provided the budwood in 1981. XCM-3 was isolated from one of 13 buds sampled (frequency, 7.7%) and XCM-4 was isolated from one bud of 49 sampled (seven buds from each of seven trees; frequency, 2%). Strain XCM-1 has been deposited with the American Type Culture Collection (ATCC), Rockville, MD, as ATCC 35393.

Bacteria were Gram negative, rod shaped (about $1.31 \times 0.53\ \mu m$), and motile by means of a single polar flagellum approximately 6 μm long (Fig. 2). The characteristics of the apple strains were those generally associated with pathovars of *X. campestris* (5–7) and were compared (Table 1). Hypersensitivity to tomato and tobacco was negative for the apple isolates but positive for *X. campestris* pv. *pruni*, a pathogen of *Prunus* spp., in companion tests. The apple strains did not induce hypersensitive reactions and were not pathogenic to cassava, peach, tomato, and grape at either inoculum level after 14 days.

Apple leaves 3 wk after inoculation with XCM-1 by airbrush did not appear different from control leaves inoculated with a heat-killed cell suspension. XCM was recovered from 19 of the 20 inoculated areas that were sampled from apple leaves even though these areas showed no visible water-soaking or necrosis.

Other bacteria isolated from the apple explants in TC were identified as *Pseudomonas* [possibly *P. cichorii* (Swingle) Stapp], *Bacillus megaterium* deBy., and *Acinetobacter* sp. Verification of bacterial identifications were provided by the ATCC (*Xanthomonas campestris*, *Bacillus megaterium*, and *Acinetobacter* sp.) and the Department of Microbiology, University of Maryland, College Park (*Pseudomonas* sp.).

Fatty acid analyses. Cellular fatty acid methyl esters of isolates of XCM (Table 2) were predominantly *iso* C15:0 (mean, 26.7%), *iso* C17:1 (22.3%), *iso* C17:0 (13.6%), C16:1$_9$ (9.5%) and *anteiso* C15:0 (7.6%). Lesser amounts of C11 and C13 and other C16 and C17 fatty acids were also detected. These fatty acids are typically produced by other pathovars of *X. campestris* (M. Sasser, *unpublished*). The presence of *iso* C11:0 3-OH, *iso* C13:0 3-OH, *iso* C15:0, and *anteiso* C15:0 fatty acids is unusual among bacteria and the ratio of *iso* C15:0 to *anteiso* C15:0 was in the range found among other *X. campestris* pathovars (M. Sasser, *unpublished*).

Propagule decontamination of surface bacteria. Removal of surface bacteria failed to result in axenic cultures of apple explants. All shoots from infected explants in the propagation medium were

TABLE 2. Cellular fatty acids of apple isolates of *Xanthomonas campestris* (XCM) and *X. campestris* pv. *pruni*

Strain	Fatty acid (%)										Ratio	
	C11:0 *iso*	C11:0 *iso* 3OH	C13:0 *iso* 3OH	C15:0 *iso*	C15:0 *anteiso*	C16:0 *iso*	C16:1 C9	C16:0	C17:1 *iso*	C17:0 *iso*	C15:0 to C15:0 *anteiso*	C17:1 *iso* to C17:0 *iso*
XCM-4	4.84	1.59	1.81	48.17	11.45	0.67	6.00	2.81	9.79	6.11	4.21	1.60
XCM-2	4.74	1.03	1.98	23.04	6.19	0.80	10.00	2.63	27.17	16.11	3.72	1.69
XCM-3	4.74	1.14	2.29	22.13	5.56	0.82	9.91	2.74	25.52	17.15	3.98	1.49
XCM-1(1)[a]	5.09	1.16	2.15	23.49	6.59	0.73	10.67	2.72	26.31	15.25	3.56	1.73
XCM-1(2)[a]	3.92	1.11	2.13	16.53	8.46	0.89	10.66	2.69	22.90	13.20	1.98	1.73
XCM mean	4.67	1.21	2.07	26.67	7.63	0.78	9.45	2.72	22.34	13.56	3.49	1.65
X. pruni mean[b]	5.22	1.87	4.42	39.21	14.18	2.15	14.47	3.05	5.23	4.85	2.77	1.08

[a] XCM-1 (1) and (2) data summarized from two experiments.
[b] Mean of nine strains of *Xanthomonas campestris* pv. *pruni*.

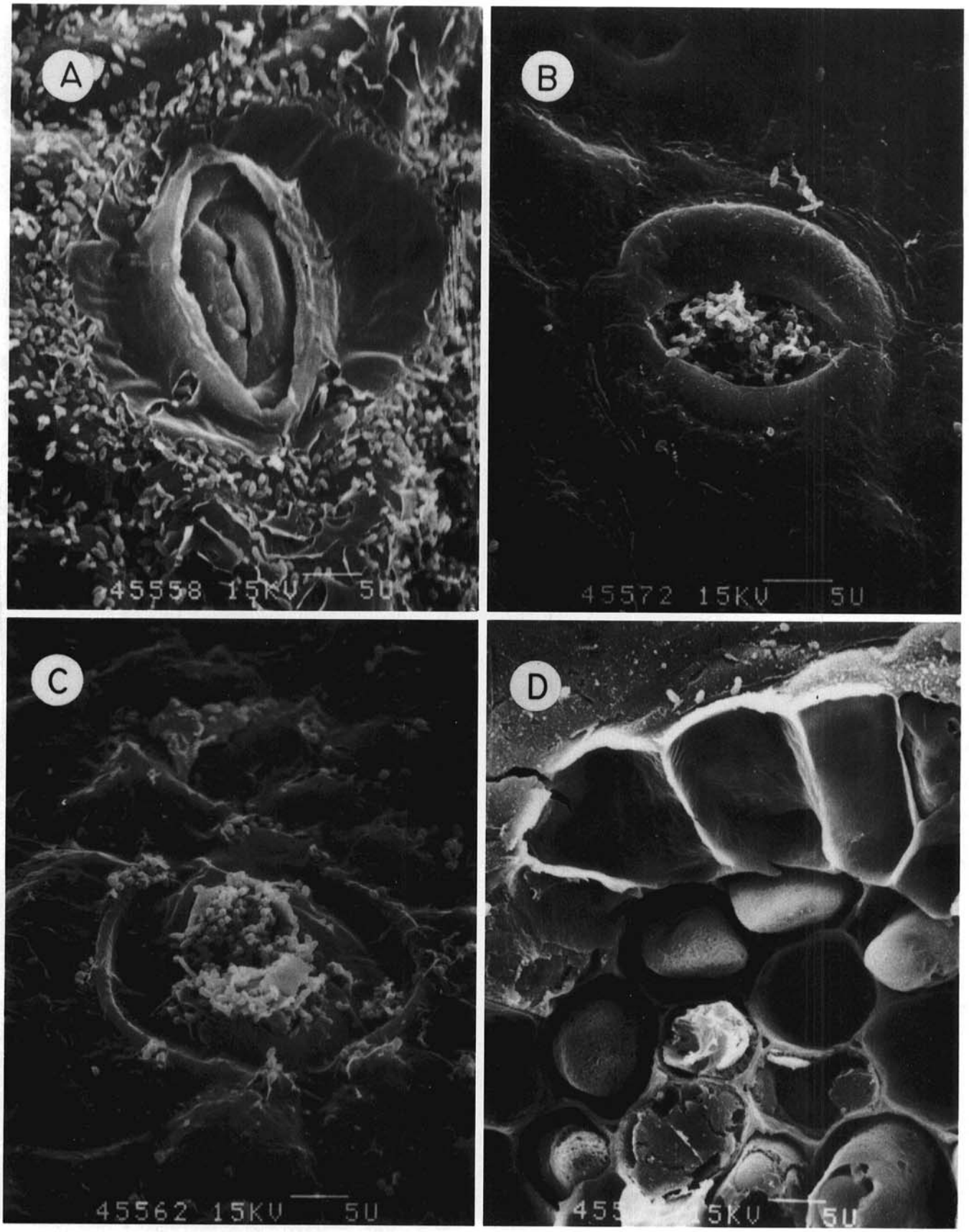

Fig. 3. Scanning electron micrographs of apple leaf tissue inoculated with *Xanthomonas campestris* (apple strain) in tissue culture. Scale bar = 5 μm. **A,** Bacterial cells on the epidermal surface appearing to erupt through the leaf cuticle and epidermis; **B,** bacterial cells in a stomatal opening; **C,** a mass of bacterial cells appearing to egress through a stoma and through the disrupted leaf cuticle; **D,** cross-section of a vein from a leaf with disease symptoms showing the absence of bacterial cells in the vein. Micrographs, courtesy of W. Wergin.

infected by XCM after treatment with the decontaminating material, suggesting that the bacteria were present internally in the apple tissue and not affected by the decontaminating procedure.

Pathogenicity tests. Inoculated explants of Redchief in the TC proliferation medium showed no leaf or stem symptoms after 30 days. However, a halo of bacterial growth (Fig. 1B) developed in the medium surrounding the submerged stem of the explants. The original explant in each culture proliferated to about 20–25 new stems after 30 days of incubation. There were no differences in numbers of new shoots from inoculated or uninoculated stems. Necrosis and water-soaked lesions developed on the leaves of many explants after 7 days of incubation in the rooting medium. Eleven of 24 and 13 of 25 explants from wound-inoculated and dip-inoculated parents, respectively, developed necrotic leaf lesions. No significant differences were found in extent of development and dry weight of infected explants compared to explants inoculated with heat-killed bacteria. XCM was reisolated from all explants propagated from inoculated parent explants.

SEM examination of apple leaf tissue infected with XCM-1 in TC showed bacteria to be present predominantly in epidermal tissues (Fig. 3A, B, and C) and not in vascular tissue (Fig. 3D). Bacteria were observed in areas of disrupted epidermal tissue and appeared to be emerging through the disrupted epidermis. Disorganized masses of bacterial cells were also seen within stomata (Fig. 3B and C), but tendrils of cells were not observed (13).

Inoculation of apple explants in the greenhouse. Symptoms of leaf infection by XCM-1 were not apparent after 2 and 4 wk following inoculation. Necrotic lesions or water-soaked areas did not appear on inoculated leaves. Areas of inoculation were apparent only as either reflective or dull circles caused by the air brush inoculation (Fig. 4A). The epidermis in the reflective spots was not disrupted; however, the epidermis was disorganized on the dull spots (Fig. 4B). In some spots from inoculated and control leaves, epidermal cells were disorganized and became necrotic, but in no case was the entire inoculated area necrotic. Damage was due to the method of inoculation rather than to infection by the bacterium. There were no differences in plant height, vigor, or color among the four cultivars or between inoculated and uninoculated plants.

XCM was isolated from several inoculated areas (Table 3) but not from water or heat-killed controls. Of the 56 inoculated areas that were cultured, only 30.4% resulted in the recovery of XCM. Cfu varied from about three to 3,390 per 28.3 mm^2 disk. There were apparent differences in frequency of recovery of XCM from among the apple cultivars that may have been due to the limited size of the experiment and to the low percent recovery of XCM.

Movement of XCM-1 in apple stems. XCM-1 was detected in one or more root systems of inoculated plants of cultivars Redspur Delicious, Paladino Spur McIntosh, Spartan, and Gala but in none of the Delicious plants (Table 4) 60 days following inoculation. There were no significant differences between methods of

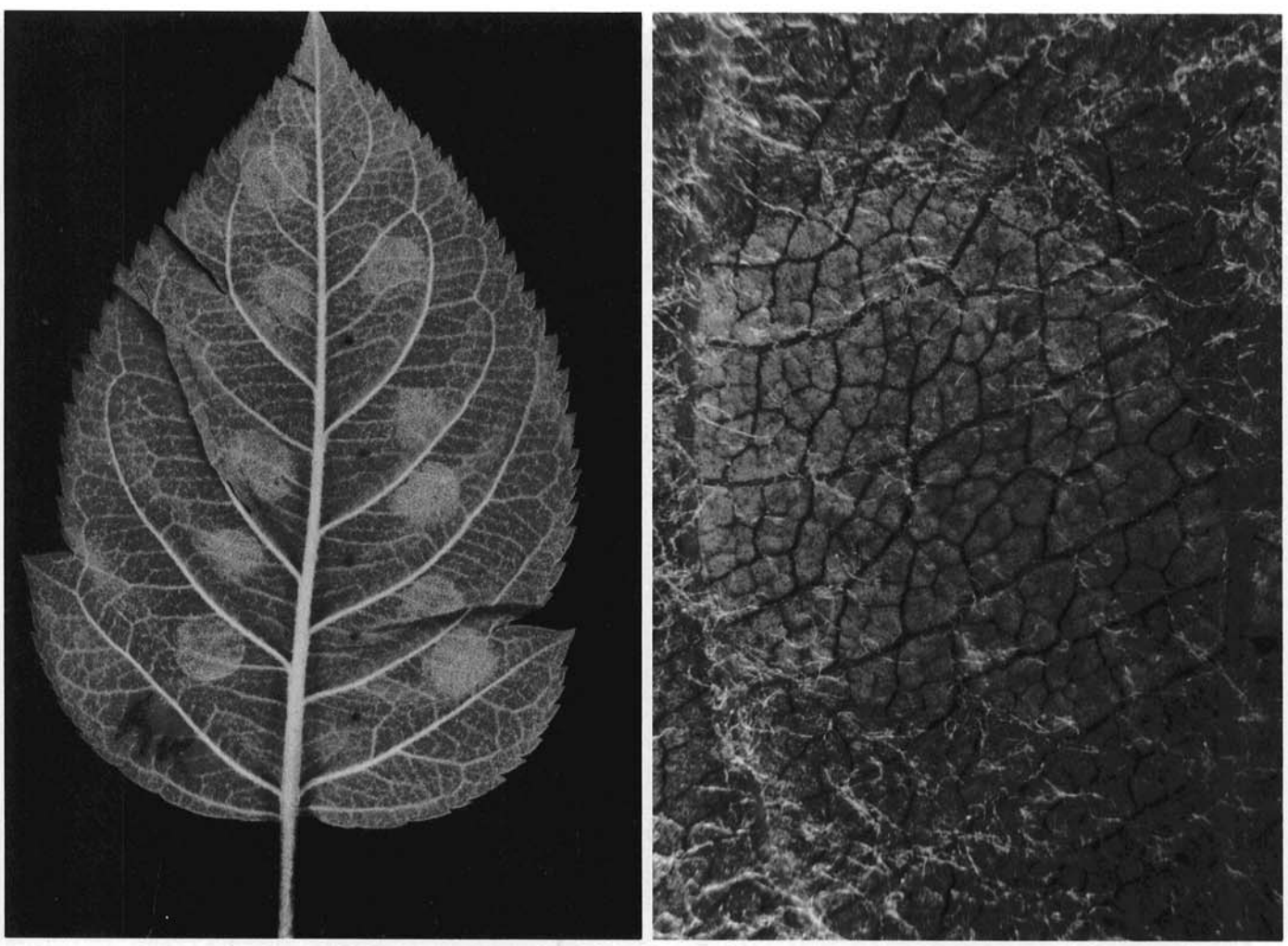

Fig. 4. Apple leaf inoculated with *Xanthomonas campestris* (apple strain) with an air brush. **A,** Right, one-half of leaf inoculated with viable cells, 10^8 colony-forming units per milliliter; left, half of same leaf inoculated with a killed cell suspension of *X. campestris* (apple strain). Light spots on the leaf were due to damage to the cuticle caused by the method of inoculation. No disease reactions were apparent 4 wk following inoculation; however, *X. campestris* (apple strain) was recovered from most of the sites inoculated with viable inoculum. Black spots on the right of the midvein are from a marker pen. **B,** Enlargement of a site on the lower surface of an apple leaf inoculated with viable bacteria.

inoculation (wounded or unwounded) and recovery of XCM-1 from roots. Percentage of root systems positive for XCM-1 was greatest among Gala plants; 88% of the wound inoculated and 100% of the dip-only inoculated root systems were positive. XCM-1 was isolated from stems of all cultivars except Gala (all treatments) and dip-inoculated Delicious and Spartan plants. The mean distances up the stems that XCM-1 was detected were 32 mm in wound-inoculated Delicious plants; 50 and 24 mm in wound- and dip-inoculated plants of Redspur Delicious, respectively; 42.7 and 31.0 mm in wound- and dip-inoculated Paladino Spur McIntosh plants, respectively; and 24.7 mm in wound-inoculated Spartan plants.

Colonization of apple leaves. Reisolation of XCM-1 was generally successful at intervals of 0–21 days following inoculation of leaves. XCM-1 was recovered from 89% of 345 inoculation sites in Experiment A and from 75% of 336 inoculation sites in Experiment B (Table 5). Reisolation of XCM-1 varied greatly between experiments and with time following inoculation. In both experiments, however, the population of XCM-1 declined sharply within 2 days following inoculation followed by an increase between 6 and 14 days following inoculation.

TABLE 3. Recovery of the apple strain of *Xanthomonas campestris* (XCM) from leaves of inoculated apple explants

Cultivar	Inoculation areas sampled[a]	XCM (cfu per three platings)[b]	Cfu total per inoculation area	Cultivar mean (positive recovery) per inoculation area
Paladino Spur McIntosh	10	18	60	
		13	43	
		6	20	
		229	763	
		6	20	181
Redchief	16	1	3	
		123	410	207
Delicious	20	1,018	3,393	
		194	647	
		112	373	
		1	3	
		7	23	
		3	10	741
Redspur Delicious	16	3	10	10

[a] Inequal sample sizes due to differences in size and availability of leaves of the cultivars. Sample area = 28.3 mm^2.

[b] Each inoculation area was macerated in 1 ml sterile distilled water, 0.1 ml aliquots were taken and spread over surface of media in petri plates (i.e., 0.1 ml per plate). Samples negative for the apple strain of *X. campestris* (XCM) are omitted from the results.

DISCUSSION

Bacteria associated with contamination and disease of apple meristem-propagated plants in tissue culture were determined on the basis of morphology, physiology, and chemical tests to be forms of *X. campestris*. Preliminary chromatographic analyses of total cellular fatty acids (as methyl esters) supported this conclusion. The genus *Xanthomonas* traditionally is comprised of phytopathogenic bacteria. The apple strains described here were associated with damage to apple explants under the special conditions of TC: presence of succulent host tissues in an exogenously induced juvenile form under conditions of high humidity. Inoculation of apple explants and other test plants with XCM-1 and growing them under greenhouse conditions failed to produce disease symptoms. XCM-1 was recovered from inoculated apple leaves up to 21 and 30 days following inoculation and from stems at various distances from inoculated roots 60 days following inoculation. Strains of XCM were able to survive in bud and leaf tissue for various periods of time and apparently were capable of limited colonization of tissues without stimulation of symptom expression. This behavior is at variance with the accepted circumscription of the genus *Xanthomonas*. Reports have been published on the occurrence of xanthomonads in asymptomatic host plants (16), as epiphytes on asymptomatic hosts (10), and in infected but presymptomatic hosts (8). In addition, *Pseudomonas maltophilia* has been determined to belong to the genus *Xanthomonas* even though it is most frequently isolated from clinical specimens, but also from soil, water, frozen foods, and plant material (21). Based on controversial evidence (20), other yellow-pigmented bacteria from clinical sources and marine habitats may be xanthomonads.

We have isolated XCM (XCM-3) from a bud of an asymptomatic apple plant that had been grown in the greenhouse for 1 yr and that was propagated from the same source of buds that yielded the initial isolate, XCM-1, in TC. XCM was also isolated in 1983 from budwood sent to Beltsville by the commercial nursery that supplied the original budwood in 1981. Based on these data, reisolation of XCM from symptomless apple leaves 21 and 30 days following inoculation with XCM-1 and reisolation of XCM from stems of apple explants 60 days following root inoculation, XCM can exist as a natural resident in apple tissue and disease symptoms are manifest only under extremely favorable conditions, such as in TC.

TABLE 4. Movement of the apple strain of *Xanthomonas campestris* (XCM) from inoculated wounded (W) and unwounded dip-inoculated (D) root systems into stems of five apple cultivars

Cultivar	Method of inoculation	Plants (no.)	Mean stem length (mm)	Mean fresh root wt. (g)	Mean stem distance with XCM (mm)	Positive stems (no.)	Root systems with XCM (no.)
Delicious	W	5	49.6	0.53	32.0	1	0
	D	5	45.6	0.60	–[a]	0	0
Redspur Delicious	W	3	50.7	0.35	50.0	1	2
	D	3	42.0	0.42	24.0	2	1
Paladino Spur McIntosh	W	5	46.0	0.27	42.7	3	2
	D	5	46.8	0.28	31.0	4	4
Spartan	W	5	28.4	0.10	24.7	3	2
	D	4	23.0	0.20	–	0	3
Gala	W	8	49.0	0.47	–	0	7
	D	10	44.3	0.28	–	0	10

[a] Minus = not detected in stem pieces.

TABLE 5. Recovery of XCM-1 at various intervals from apple leaves inoculated with 10^8 and 10^6 cfu

Initial inoculum concentration (no./ml)	Time from inoculation (days)	Sites inoculated (no.)	Cfu per site	
			Range (no.)	Mean (no.)
10^8	0	8	5–11,000	2,857
	1	8	0–413	134
	2	8	1–588	140
	3	8	0–951	307
	4	8	0–1,252	223
	7	8	1–1,207	168
	10	8	0–3,472	683
	14	8	44–4,471	1,722
10^6	0	12	0–791	224
	1	12	0–398	99
	3	12	0–123	43
	7	12	2–357	161
	10	12	27–598	189
	14	6[a]	0–4,150	850
	21	6[a]	34–366	176

[a] This set of inoculation sites was split numerically to extend the experiment from 14 to 21 days.

We are not aware of any other naturally occurring disease of apple caused by *Xanthomonas* nor of any other asymptomatic relationships between pathovars of *X. campestris* and apple. Several surveys of apple bud and leaf microflora have not indicated the presence of xanthomonads (1,2,12). It is significant that a xanthomonad can exist asymptomatically in apple tissue and be transmitted by TC-propagation. Presumably XCM could also be transmitted by traditional means of vegetative propagation; i.e., grafting of budwood onto rootstock. The implications of the asymptomatic presence of XCM in mature apple tissue is not fully apparent. However, overwintering or survival of some bacteria (e.g., *X. campestris* pv. *juglandis*) in apparently healthy buds, during periods unfavorable for growth or infection is an important factor in their survival and epidemiology and especially for pathogens that have no known alternate host (16). We consider these apple strains to represent an unusual form of *X. campestris*. It is possible that apple is not the primary host for XCM, although attempts to inoculate several other hosts failed to result in production of symptoms. If these strains of XCM are natural inhabitants of apparently healthy apple bud material and if they have no other host in which they cause a disease, a reexamination of the traditional requirement that all xanthomonads are plant pathogens may be in order (18).

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